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14. ABSTRACT This is a final report of Pre-doctoral Prostate Cancer Training Program (PCRP) for Dr. Serk In Park, a Ph.D. student in the Program of Cancer Biology, the University of Texas M.D. Anderson Cancer Center. Dr. Park, the PI of this training grant, has completed all requirements for the degree of Ph.D. earlier than the originally proposed date, leading to early termination of the grant. This final report includes scientific and educational accomplishments directly supported by this grant, and key findings of scientific research.				
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusion.....	6
References.....	7
Appendices.....	7

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in men in the USA (1). The survival rate decreases drastically when metastases, including those to the bone and lymph nodes, are found at the time of diagnosis (2). Thus, novel therapeutic strategies that target molecules involved in early stages of the metastatic process will be beneficial to the survival of prostate cancer patients. Among many biological changes contributing to the prostate cancer progression to metastasis, activation of Src family kinases (SFK), a class of protein tyrosine kinases, plays critical roles in regulating molecular changes in both tumor cells and cells in the tumor microenvironment (3). Accordingly, drugs targeting SFK have recently advanced to clinical trials for prostate cancer (4, 5). Better understanding of the function and molecular mechanism of SFK-mediated biological functions will provide insights into the mechanism of lymph node metastasis and also provide guidance for the application of SFK inhibitors to the prostate cancer treatment.

The subject of scientific research in this training grant is to study the roles of SFK in interactions between tumor and microenvironment, leading to development of prostate cancer lymph node metastasis. The scope of research covers from laboratory findings to translational application of those findings into clinic. Additionally, this competitive training grant will teach the PI the scientific principles in developing and testing specific hypothesis with respect to development of prostate cancer lymph node metastases. The PI will also gain insights into the use of animal models, and learn aspects of cell and molecular biology required to complete the project. These insights should guide the PI through a successful career track to an independent researcher in the field.

BODY

During the period of this training grant support from July 1, 2008 to September 13, 2008, the PI has successfully defended his dissertation work in a public seminar given to a general scientific audience and the Ph.D. supervisory committee (chaired by Dr. Gary E. Gallick, Ph.D., Professor of Cancer Biology, the University of Texas M. D. Anderson Cancer Center). The committee recommended the PI's Ph.D. degree in Cancer Biology, which was approved by the dean of the Graduate School of Biomedical Sciences at the University of Texas M.D. Anderson Cancer Center. The dissertation work was partly supported by this training grant, especially the last semester of the dissertation work, and is thus indicated in the acknowledgement page of the dissertation.

With the Ph.D. degree and the extensive training in cancer biology, partly supported by this training grant, the PI was offered a postdoctoral research fellow position in several competitive academic institutions and a federal government of the U.S. The PI has accepted an offer from the University of Michigan, and started the position on September 22, 2008. During post-doctoral research fellowship in the University of Michigan, the PI will focus on the roles of parathyroid hormone-related peptide (PTHrP) in prostate cancer bone metastasis, which is a logical extension of but distinct from the PI's dissertation studies.

Because the PI completed all of the requirements for the degree of Ph.D. degree much earlier than the timeframe proposed in the original training grant proposal, funding will be terminated early. The total period of grant support was only 75 days including holidays, resulting in limited

research accomplishments directly associated with the tasks outline in the approved Statement of Work, but did include some work that would not otherwise have been performed..

However, the scientific research accomplishments supported by this grant provide crucial answers to the cancer biology of prostate cancer, and also critical rationale for the clinical application of novel drugs currently in clinical trials. Among those key findings in the PI's dissertation research, the PI has reported for the first time that, in prostate cancer, two Src family kinase members (Src and Lyn) play distinctive roles in prostate cancer cells, with Src promoting metastasis and Lyn promoting proliferation. In addition, the PI has provided the first direct experimental evidence in his dissertation that pharmacological inhibition of SFK significantly reduced prostate tumor growth and development of lymph node metastasis in preclinical orthotopic nude mouse models. Therefore, the PI has established the biological rationale of SFK inhibitors in clinical trials for their efficacy in prostate cancer patients, suggesting that SFKs are promising therapeutic targets for prostate cancer (6).

In addition to his dissertation, the PI has prepared a manuscript as the first author, currently in submission for publication in a scientific journal. The manuscript provides crucial scientific evidence for application of the application of a novel drug in the metastatic prostate cancer patients. The PI tested the effects of a novel SFK inhibitor, recently entered the Phase I clinical trials in M. D. Anderson Cancer Center, on growth of prostate cancer and development of lymph node metastasis in a human prostate cancer mouse model. For the details of the key findings, manuscript is attached to this final report as an appendix.

KEY RESEARCH ACCOMPLISHMENTS

- Two members of the Src family kinases, Src and Lyn, affect distinct stages of prostate tumor growth and lymph node metastasis.
- Inhibition of SFKs *in vivo* leads to decreased cellular proliferation and increased apoptosis.
- Dasatinib treatment effectively inhibits expression of activated SFKs (Src and Lyn) *in vitro* and *in vivo*.
- Dasatinib, a SFK inhibitor in clinical trial, inhibits the primary tumor growth and lymph node metastasis in an orthotopic nude mouse model.
- KX2-391, a novel SFK inhibitor targeting peptide-binding domain of SFK, inhibits the primary tumor growth and lymph node metastasis in an orthotopic nude mouse model.
- SFKs are promising therapeutic targets for treatment of human prostate cancer.

REPORTABLE OUTCOMES

1. A Ph.D. degree obtained from the Graduate School of Biomedical Sciences, the University of Texas M.D. Anderson Cancer Center, Houston, TX. The doctoral dissertation work entitled “Role of Src Family Kinase Activation in Prostate Cancer Lymph Node Metastasis” was partly supported by this award.
2. Employment as a postdoctoral research fellow in the University of Michigan, based on works partly supported by this award.
3. A manuscript prepared and submitted for publication in a scientific journal: “A Novel Src Family Kinase Inhibitor Targeting Peptide Substrate Binding Domain (KX2-391) Inhibits Prostate Cancer Growth and Lymph Node Metastasis *in vivo*.” Works in this manuscript were partly supported by this training grant, and manuscript was prepared in collaboration with Dr. Gary E. Gallick, Ph.D., the mentor of the PI on this training grant.

CONCLUSION

Aberrant expression/activity of Src family kinases occur frequently in multiple types of human cancers, including prostate cancer. In prostate cancer, two SFK members, Src and Lyn, have been specifically implicated in tumor growth and progression. The PI has demonstrated in his dissertation, partly supported by this training grant, that Src and Lyn play distinctive roles in prostate cancer cells, with Src promoting metastasis and Lyn promoting proliferation. In addition, the PI demonstrated that pharmacological inhibition of SFK significantly reduced prostate tumor growth and development of lymph node metastasis in preclinical orthotopic mouse models. Therefore, studies in this training grant established the biological rationale of SFK inhibitors in clinical trials for their efficacy in prostate cancer patients, suggesting that SFKs are promising therapeutic targets for prostate cancer, and was important in bringing one of these inhibitors, KX2-391, into Phase I clinical trials at the M.D. Anderson Cancer Center and Roswell Park Cancer Institute. In addition, the understanding of the unique effects of SFK inhibitors in prostate cancer has led to prostate cancer being the first tumor type selected for an international Phase III trial using dasatinib plus docetaxel. Thus, the continued combination of molecular and translational studies remains critical to basic mechanistic understanding of roles of SFKs, and optimum use of SFK inhibitors in cancer therapy.

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APPENDICES

1. Biosketch of the PI.
2. Title and abstract pages of Ph.D. dissertation (A complete microfilm copy of dissertation has been deposited in the library of the University of Texas and the U. S. Library of Congress for public access)
3. Manuscript submitted for publication in a scientific journal.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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1. Park YG, Park S, Lim SO, Lee MS, Ryu CK, Kim I, Cho-Chung YS. Reduction in Cyclin D1/Cdk4/Retinoblastoma protein signaling by CRE-decoy oligonucleotide. Biochem Biophys Res Commun. 2001; 281:1213-9.
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U.S. Department of Defense
Role of c-Src Activation on Prostate Cancer Lymph Node Metastases
The major goal of this training grant is to study role of Src family kinase in prostate cancer progression
Role: PI

**THE ROLE OF SRC FAMILY KINASE ACTIVATION IN
PROSTATE CANCER GROWTH AND
LYMPH NODE METASTASIS**

By

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**THE ROLE OF SRC FAMILY KINASE ACTIVATION IN
PROSTATE CANCER GROWTH AND
LYMPH NODE METASTASIS**

A

DISSERTATION

Presented to the Faculty of

The University of Texas
Health Science Center at Houston

And

The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY
in Cancer Biology

by

Serk In Park, D.D.S., M.S.
Houston, Texas

December 2008

DEDICATION

To my family, for their continual support and devotion.

To my teachers at each level of education, for their instruction and encouragement.

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I express my deepest appreciation to my advisor, Dr. Gary E. Gallick, for his persistent patience, guidance and encouragement.

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I wish to thank the Vivian L. Smith Foundation, the U. S. Department of Defense, and the Graduate School of Biomedical Sciences, for their financial support.

THE ROLE OF SRC FAMILY KINASE ACTIVATION IN PROSTATE CANCER GROWTH AND LYMPH NODE METASTASIS

Publication No. _____

Serk In Park, D.D.S., M.S.

Supervisory Professor: Gary E. Gallick, Ph.D.

Aberrant expression and/or activation of Src Family of non-receptor protein tyrosine kinases (SFKs) occur frequently during progressive stages of multiple types of human malignancies, including prostate cancer. Two SFKs, Src and Lyn, are expressed and implicated in prostate cancer progression. Work in this dissertation investigated the specific roles of Src and Lyn in the prostate tumor progression, and the effects of SFK inhibition on prostate tumor growth and lymph node metastasis in pre-clinical mouse models.

Firstly, using a pharmacological inhibitor of SFKs in clinical trials, dasatinib, I demonstrated that SFK inhibition affects both cellular migration and proliferation *in vitro*. Systemic administration of dasatinib reduced primary tumor growth, as well as development of lymph node metastases, in both androgen-sensitive and -resistant orthotopic prostate cancer mouse models. Immunohistochemical analysis of the primary tumors revealed that dasatinib treatment decreased SFK phosphorylation but not expression, resulting in decreased cellular proliferation and increased apoptosis. For this analysis of immunohistochemical stained tissues, I developed a novel method of quantifying immunohistochemical stain intensity that greatly reduced the inherent bias in analyzing staining intensity.

To determine if Src and Lyn played overlapping or distinct roles in prostate cancer tumor growth and progression, Src expression alone was inhibited by small-interfering RNA. The resulting stable cell lines were decreased in migration, but not substantially affected in proliferation rates. In contrast, an analogous strategy targeting Lyn led to stable cell lines in which proliferation rates were significantly reduced.

Lastly, I tested the efficacy of a novel SFK inhibitor (KX2-391) targeting peptide substrate-binding domain, on prostate cancer growth and lymph node metastasis *in vivo*. I demonstrated that KX2-391 has similar effects as dasatinib, an ATP-competitive small molecular inhibitor, on both the primary tumor growth and development of lymph node metastasis *in vivo*, work that contributed to the first-in-man Phase I clinical trial of KX2-391.

In summary, studies in this dissertation provide the first demonstration that Src and Lyn activities affect different cellular functions required for prostate tumor growth and metastasis, and SFK inhibitors effectively reduce primary tumor growth and lymph node metastasis. Therefore, I conclude that SFKs are promising therapeutic targets for treatment of human prostate cancer.

TABLE OF CONTENTS

Approval Sheet	i
Title Page	ii
Dedication	iii
Acknowledgement	iv
Abstract	v
Table of Contents	vii
List of Illustrations	viii
List of Tables	xi
Chapter 1: Introduction	1
Chapter 2: Materials and Methods	35
Chapter 3: Differential Roles of Two Src Family Kinases, Src and Lyn, in Prostate Cancer Cells <i>in vitro</i>	47
Chapter 4: The Effects of Pharmacologic Inhibition of Src Family Kinases on Prostate Cancer Growth and Development of Macroscopic Lymph Node Metastasis <i>in vivo</i>	64
Chapter 5: Development of a Novel Method for Quantitative Analysis of Immunohistochemical Stain Intensity	81
Chapter 6: The Effects of a Novel Src Family Kinases-Selective Inhibitor on Prostate Cancer Growth and Development of Lymph Node Metastasis <i>in vivo</i>	90
Chapter 7: Discussion	101
Bibliography	123
Vita	155

LIST OF ILLUSTRATIONS

Figure 1	Prostate Cancer Progression	18
Figure 2	Structural Domains of Src Family Kinases	21
Figure 3	Activation of Src	23
Figure 4	Src-Mediated Pathways Contributing to Homeostasis and Tumor Progression	24
Figure 5	SFK Activation during Prostate Progression	27
Figure 6	Dasatinib Effects on Src, FAK and p130Cas Phosphorylation	50
Figure 7	Time-Course of Dasatinib Effects on Inhibition of Src and FAK Phosphorylation	51
Figure 8	Effects of Dasatinib on Src and FAK Phosphorylation in LNCaP and PC-3 cells	52
Figure 9	Effects of Dasatinib on Inhibition of Lyn Phosphorylation	54
Figure 10	Time-Course of Dasatinib-Induced Inhibition of Lyn Phosphorylation	55
Figure 11	Effects of Dasatinib on Proliferation of PC-3MM2GL Cells <i>in vitro</i>	57
Figure 12	Effects of Dasatinib on Proliferation of LNCaP Cells <i>in vitro</i>	58
Figure 13	Effects of Dasatinib on Migration of PC-3MM2GL Cells in Culture	59
Figure 14	Generation of <i>Src</i> - and <i>Lyn</i> -specific Knockdown Clones	60
Figure 15	Effects of <i>Src</i> - or <i>Lyn</i> -specific Knockdown on Proliferation of PC3-MM2GL Cells	62
Figure 16	Migration of <i>Src</i> -specific Knockdown Clones	63
Figure 17	Determination of tumor cell engraftment by <i>in vivo</i> bioluminescence imaging	68

Figure 18	Necropsy Pictures of Control- and Dasatinib-Treated Mice	70
Figure 19	<i>In vivo</i> Effects of Dasatinib on Growth	71
Figure 20	Immunohistochemistry of Src, Lyn and Autophosphorylated SFKs in Primary Tumor Tissue	74
Figure 21	Immunohistochemistry of Tumor Tissue for FAK and FAK Phospho-[Y ⁸⁶¹]	76
Figure 22	Immunohistochemistry of Tumor Tissues for Proliferating Cell Nuclear Antigen (PCNA)	77
Figure 23	Immunohistochemistry of Tumor Tissues for TdT-mediated dUTP nick-end labeling (TUNEL) Assay	78
Figure 24	Immunohistochemistry of Tumor Tissues for CD31	80
Figure 25	Potential Selection Bias of Standard Quantification of Immunohistochemistry Staining	83
Figure 26	Color Selection Bias of Immunohistochemistry Staining Quantification	84
Figure 27	Algorithm of a Novel Quantification Macro for IHC staining	86
Figure 28	An Example of the Automated Quantification	89
Figure 29	Immunohistochemistry of Tumor Tissue for Src, Lyn and Autophosphorylated SFK expression	95
Figure 30	Quantification of Expression of Autophosphorylated SFKs by Immunohistochemistry Staining Intensity	96
Figure 31	Immunohistochemistry of Tumor Tissues for Proliferating Cell Nuclear Antigen (PCNA)	99
Figure 32	Immunohistochemistry of Tumor Tissues for CD31	100
Figure 33	Schematic Representation of the Hypothesized Mechanism	120

LIST OF TABLES

Table 1	Effects of Dasatinib on Growth and Lymph Node Metastasis of PC-3MM2GL or PC-3AR-A1 Cells in an Orthotopic Nude Mouse Model	67
Table 2	Primary Tumor Size does not Affect Development of Lymph Node Metastases	73
Table 3	Effects of KX2-391 on Growth and Lymph Node Metastasis of PC-3MM2GL Cells in an Orthotopic Nude Mouse Model	93

A Novel Src Family Kinase Inhibitor (KX2-391) Targeting the Peptide Substrate Binding Domain Inhibits Prostate Cancer Growth and Lymph Node Metastasis *in vivo*

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ABSTRACT

Four small molecule inhibitors of SFK/Abl are currently in early phase clinical trial for numerous solid tumors. Of these, KX2-391 is novel in that it competes with the peptide binding region of Src as opposed to ATP competition (the mechanism of action of the other. As of yet, no preclinical animal studies have examined efficacy of this new class of SFK inhibitors. To determine the efficacy of KX2-391, we conducted *in vivo* orthotopic mouse experiments in prostate cancer, and compared the effects with dasatinib. For these studies, a highly metastatic variant of human prostate cancer cells, PC-3MM2GL, was surgically implanted into the prostate of male nude mice. After three days, animals were randomized into four groups receiving placebo, low dose (5mg/kg/day) or high dose (10mg/kg/day) of KX2-391, or dasatinib (15mg/kg/day), respectively. At the end of 28-day treatment, mice were killed and examined for the primary tumor weight and the presence of lymph node metastases. Tumors were then harvested and analyzed by immunohistochemistry. KX2-391 (10mg/kg/day) treatment significantly reduced primary tumor weight and incidence of lymph node metastasis, compared to the placebo, and the effects were similar as 15mg/kg/day dasatinib treatment. Immunohistochemistry demonstrated that KX2-391 effectively inhibits activating phosphorylation of Src family kinases, and also reduces tumor cell proliferation *in vivo*. These results demonstrate that KX2-391 is a promising therapeutic agent for treatment of prostate cancer, and may be of efficacy when other SFK inhibitors show toxicity..

INTRODUCTION

The Src family of non-receptor protein tyrosine kinases (SFKs) plays numerous regulatory roles in development and homeostasis-MAYBE PUT A LITTLE MORE HERE WITH REFS, and are frequently activated in solid and hematologic malignancies during tumor progression and metastasis. Recently, a surge of interest in SFK-selective inhibitors has prompted nearly early stage clinical trials. Four SFK inhibitors are now in various stages of trial as anti-cancer therapeutics. Dasatinib (BMS-354825) is a potent SFK inhibitor that is now approved by the U. S. Food and Drug Administration for treatment of Philadelphia chromosome-positive chronic myelogenous leukemia (CML) and acute myelogenous leukemia (14, 15). In addition, dasatinib is currently in Phase I/II clinical trials for solid tumors including prostate cancer, and soon will enter multinational Phase III clinical trials (BMS trial CA180227). However, dasatinib may not be effective for all patients for a few reasons. Firstly, in phase I/II clinical trials for imatinib-resistant CML patients, dose-limiting toxicity has occurred in some patients due to pleural effusion (16). The SFK inhibitors AZD0530 and SKI606 are also in clinical trial for several tumors...Common side effects of these inhibitors are GI... Most of these trials remain in early stages, precluding determination of their ultimate success at this time. As protein tyrosine kinases share close structural homology and consequently kinase inhibitors targeting kinase domains (such as ATP-competitive inhibitors) show multiple spectra of inhibition. Indeed, dasatinib has been shown to affect BCR-ABL, c-Kit, platelet-derived growth factor receptor (PDGFR) and EphA2 in higher concentrations, and some of these targets may account for some of the toxicities seen with these inhibitors.

In this report, we study KX2-391, the prototype of a new class of Src inhibitors. KX2-391 (formerly KX-01) is a novel, potent and water-soluble SFK inhibitor, targeting peptide-binding domain of SFK (AACR REF). It is currently in phase I/II clinical trials for human malignancies including PCa. However, questions regarding whether this novel drug would have similar or better efficacy compared to other ATP-competitive Src inhibitors in preclinical animal models have yet to be determined. We have chosen to examine the efficacy of KX2-391 in orthotopic nude mouse models for prostate cancer.

Prostate cancer models were chosen for multiple reasons. Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-related death in American men (1). Similar to many other types of solid tumors, distant metastases, most frequently to bones and lymph nodes, are major cause of mortality and morbidity of the patients. Particularly, lymph node metastasis has been shown to be closely associated with poor prognosis (2-5). In addition, extended pelvic lymph node dissection increases progression-free survival of the patients at high risk of lymph node involvement (6, 7). Therefore, therapeutic strategies that would inhibit growth of existing lymph node metastases or prevent development of lymph node metastases would greatly improve survival of patients with lymph node-positive prostate cancer. In addition, because Src effects osteoclast function, Src inhibitors are hoped to be useful in the treatment of bone mets (fill in a little more with refs). It is for these reasons that a multicenter phase III trial of dasatinib + docetaxel will be initiated in 2009. Work in our laboratory in orthotopic nude mouse models has demonstrated that dasatinib is effective in inhibiting both tumor proliferation (through Lyn inhibition) and independently, metastasis to the lymph node. For these reasons, we utilized the same orthotopic model to compare efficacy of these two classes of Src inhibitors; dasatinib, an ATP competitor, and KX2-391, a competitor of Src interactions. We report that in this preclinical model, dasatinib and KX2-391 show similar efficacy, suggesting that peptide binding inhibitors of Src and other tyrosine kinases may

MATERIALS AND METHODS

Cells and Cell Culture

The highly metastatic variant of PC-3 (PC-3MM2) was established by several cycles of *in vivo* orthotopic implantation-metastatic selection, and the cells were further modified to express a green fluorescent protein and luciferase (GL) fusion gene for *in vivo* bioluminescence imaging, as previously described (11, 17). Cells were maintained as monolayer cultures in DMEM/Ham's F-12 media supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin (Gibco® Invitrogen Co.), and incubated in 5% CO₂/95% air at 37°C. Cultures were free of mycoplasma and murine viral pathogens (assayed by Whittaker M. A. Bioproducts).

Orthotopic implantation of tumor cells

Orthotopic prostate tumor model was established as previously described (11, 18). Briefly, PC-3MM2GL cells were detached from subconfluent cultures and a desired number of cells were centrifuged and resuspended with Ca²⁺- and Mg²⁺-free Hank's Balanced Salt Solution (Gibco® Invitrogen Co.) Forty male athymic nude mice (NCR-nu/nu; 8 to 12 weeks of age; the National Cancer Institute-Frederick Animal Production Area) were anesthetized with pentobarbital sodium i.p. (0.5mg per 1g body weight) (Nembutal®, Abott Laboratories), and placed in a supine position. A midline incision was made on the lower abdomen and the prostate was exteriorized. Fifty microliters of HBSS containing PC-3MM2GL (5×10^4 cells) cells were injected into the dorso-lateral side of the prostate. The incision was closed with surgical metal clips. The animals were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U. S. Department of Agriculture, the U. S. Department of Health and Human Services, the U. S. National Institutes of Health and the University of Texas M. D. Anderson Cancer Center institutional guidelines.

Drug formulation and administration

Three days after xenograft injection, mice were randomized into four groups ($n=10$ each group) and tumor engraftment was determined by *in vivo* bioluminescence imaging (IVIS™ 100 bioluminescence imaging system, Xenogen Co.) under 2% isoflurane inhalation anesthesia. Six mice per group showing luciferase activity close to the median value were selected for treatment. Mice received control vehicle, KX2-391 (5.0 or 10.0 mg/kg body weight/day) or dasatinib (15mg/kg body weight/day). For oral administration, KX2-391 (Kinex Pharmaceuticals) was dissolved in double-distilled H₂O and dasatinib (synthesized by Kinex Pharmaceuticals) was dissolved in 80mM citrate buffer (pH=3.1) according to the manufacturer's instruction. Total daily dosage of drug was divided by two and administered p. o. at 12-hour intervals, using 20-gauge gavage needle. Control group mice were administered with the equal volume of water by the same gavage technique. Mice were treated for 28 days.

Necropsy and tissue preparation

At the end of four-week's treatment, mice were euthanized by pentobarbital sodium overdose (1mg per 1g body weight) four hours after the last drug dose or control diluent was administered. Lymph node metastasis was assessed macroscopically and enlarged lymph nodes were harvested for pathologic examination. Tumors were surgically excised and weighed, followed by fixation in phosphate-buffered 10% formaldehyde. A part of tumor tissue was embedded in O.C.T. compound (Sakura Finetek), snap-frozen in liquid nitrogen, and stored at -80°C. Three medium-sized tumors from each group were chosen for further immunohistochemical analysis.

Immunohistochemical staining of total Src, Lyn, FAK and autophosphorylated SFKs, and FAK-phospho-[Y⁸⁶¹]

Immunohistochemistry was performed as previously described (11). Briefly, paraffin-embedded tumor tissues were sectioned 8-10μm thickness and mounted on positively charged microscope slides. Antigen retrieval was accomplished either by boiling the slides in pressure cooker at 125°C for five minutes, immersed in Borg decloaker solution (Biocare Medical Inc.) for Src, autophosphorylated SFKs and Lyn

staining; or by boiling the slides in 0.1M EDTA buffer for five minutes using a microwave oven and subsequently incubating the slides for one hour in Dako target retrieval solution (Dako North America Inc.) for FAK and FAK-phospho-[Y⁸⁶¹] staining, respectively. Dilution of primary antibodies are as follows: anti-Src antibody (1:100, Cell Signaling Technology Inc.), anti-phospho-[Y⁴¹⁶]-Src antibody (1:100, Cell Signaling Technology Inc.), anti-Lyn antibody (1:100, SC-15, Santa Cruz Biotechnology Inc.), anti-FAK antibody (1:100, Cell Signaling Technology Inc.) and anti-phospho-[Y⁸⁶¹]-FAK (1:100, Biosource™ Invitrogen Co.). Mach 4 Universal HRP polymer (Biocare Medical Inc) was used as a secondary antibody. The stain was visualized by incubation in 3, 3'-diaminobenzidine (DAB) and counterstain with Gill's No. 3 hematoxylin. Internal negative control samples were exposed to protein block solution instead of the primary antibodies and demonstrated no specific signaling. Slides were dried and mounted with Universal Mount solution (Research Genetics, Invitrogen Co.).

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA)

To determine the percentage of proliferating cells, paraffin-embedded sections were stained for PCNA, as previously described (19). For quantification, three randomly selected bright-field microscope images (40× magnification; 0.89mm² area) per sample were obtained as described above. The total cell number in each image was calculated by counting hematoxylin-positive cells using ImageJ's particle count command, and DAB-positive cells were counted as PCNA-positive cells in each image.

Statistics

All statistical analyses were performed in SPSS 12.0 for windows (SPSS Inc., Chicago, IL). The Mann-Whitney *U* test was conducted to compare differences in tumor weight. Incidences of tumors and lymph node metastases were compared between groups with Fisher's exact test. PCNA-positive cells were compared by Student's *t*-test. All statistical tests were two-sided.

RESULTS

Oral administration of KX2-391 shows similar efficacy as dasatinib in reducing *in vivo* orthotopic tumor weight and incidence of lymph node metastases.

To examine the effects of KX2-391 on growth of the primary tumor and/or development of lymph node metastases in comparison to dasatinib, a highly metastatic variant of PC-3 cell line, PC-3MM2GL, was used to establish an orthotopic prostate cancer nude mouse model. Cells (1×10^5 cells in 50 μ l Hank's balanced salt solution) were injected into the prostates of nude mice ($n=40$). After three days, mice were randomly assigned into four groups ($n=10$, each), and tumor cell engraftment was determined by *in vivo* bioluminescence imaging. In each group, six animals emitting bioluminescence signals close to the median value were chosen, to reduce tumor size variance within each group. Mice were treated with control buffer (distilled water), low dose (5mg/kg/day) or high dose (10mg/kg/day) of KX2-391, and dasatinib (15mg/kg/day) for 28 days by oral gavage. Daily dosage was divided by two and administered in 12-hour intervals. Mice were sacrificed four hours after the last dosage of four-week treatment. Tumor incidences among four groups were not significantly different (**Table 1**). Tumors from mice receiving high dose KX2-391 were significantly reduced in weight compared to the control (**Table 1 and Figure 1**, $P=0.016$, Mann-Whitney *U* test). Tumors from mice receiving low dose KX2-391 were smaller than the control tumors in weight, but the reduction was not statistically significant ($P=0.076$, Mann-Whitney *U* test). Tumors from the dasatinib-treated group tumors were significantly reduced in weight compared to the control ($P=0.028$, Mann-Whitney *U* test), but there was no statistical significance when compared to both high- and low- dose KX2-391-treated group tumors. Furthermore, high dose KX2-391 or dasatinib administration significantly reduced the incidence of metastases to the iliac lymph nodes (**Table 1**, $P=0.015$ for both, Fisher's exact test), compared to the control diluent-treated mice. Lymph node metastases were determined macroscopically, as previously described (11), and metastatic lymph nodes were harvested and pathologically confirmed by hematoxylin and eosin staining.

KX2-391 treatment decreases expression of autophosphorylated SFKs in tumors.

To determine whether KX2-391 effectively inhibited activation of SFKs in the primary tumors *in vivo*, tumors tissues were harvested, fixed and prepared for immunohistochemical analysis as described in the Materials and Methods. Area-matched representative pictures were shown in **Figure 2**, and intensity of phospho-SFK staining was quantified. Dasatinib-treated tumors had significantly reduced level of phospho-SFK expression, compared to the control tumors ($P<0.01$, Student's t-test) (**Figure 3**). Furthermore, both low dose (5mg/kg/day) and high dose (10mg/kg/day) KX2-391-treated tumors had significantly decreased phospho-SFK expression level compared to the control (both $P<0.01$, Student's t-test), suggesting that KX2-391 targeting peptide-binding domain of SFKs also inhibits activating autophosphorylation of SFKs. Interestingly, high dose KX2-391 treated tumors showed significantly higher staining intensity than low dose-treated tumors ($P<0.01$, Student's t-test). Nonetheless, staining intensity of phospho-SFK in dasatinib-treated tumors was not statistically different in either the low dose KX2-391-treated tumors ($P=0.18$, Student's t-test) or in the high dose KX2-391 treated tumors ($P=0.07$, Student's t-test), suggesting that both concentrations of KX2-391 treatment had similar efficacy in suppressing activation of SFKs in preclinical prostate tumor tissues, at least in this assay.

KX2-391 administration reduces number of proliferating cells in tumor tissues.

As shown in previous works (11), dasatinib had anti-proliferative effects on human prostate cancer cells *in vitro*; thus we determined the effects of dasatinib on tumor cell proliferation *in vivo* by proliferating cell nuclear antigen staining. As shown in **Figure 4**, both high- and low-dose KX2-391 treatment decreased percentage of PCNA-positive tumor cells significantly (both $P<0.05$, Student's t-test), compared to the control treatment (**Figure 4, A**). Dasatinib treatment also reduced the percentage of PCNA-positive tumor cells, compared to the control ($P<0.05$, Student's t-test). However, percentage of proliferating cells in dasatinib-treated tumors was not different from those of both KX2-391 groups (both $P>0.05$, Student's t-test).

To determine whether tumor growth inhibition of KX2-391 treatment could also be attributed to inhibition of angiogenesis, microvessel densities were measured by counting CD31/PECAM-positive microvessels. Microvessel densities among all four

groups were not significantly changed (**Figure 5**, $P>0.5$, Student's t-test). In summary, similar to the dasatinib data (11), our results demonstrate that KX2-391 treatment of mice bearing prostate tumors results both in decreased growth rate and decreased metastatic potential to the lymph nodes.

DISCUSSION

While one of the promises of signal transduction inhibitors (STIs) is that they were likely to be less toxic than standard chemo-therapies, such has not always proven the case. For example, pleural effusion has been associated with dasatinib, as well as GI complications, among others (22).

Each of the above agents has taken the now standard approach of designing small molecule inhibitors that compete with ATP for binding to the kinase. Such inhibitors show modest selectivity, and often target numerous other kinases at various IC₅₀s, some of which may be relevant to normal and tumor functions. Src family kinases present a distinct potential problem in this area, as ATP competitive inhibitors generally inhibit most if not all members of the family at relatively low IC₅₀s in the nanomolar range, and the highly diverse functions of Src would lead to the expectation that SFK inhibitors might be toxic. It is therefore surprising that, in current trials, toxicity has often not been a major problem.

Nevertheless, Src inhibitors that might affect selected functions of Src (e.g. interactions with proteins) rather than kinase represent an exciting class of novel inhibitors with unique potentials as therapeutics. KX2-391 represents the first compound in its class. Not only is it structurally and functionally unique (see **Fig. 1**), it has been shown to be potent as an inhibitor in several *in vitro* models. However, no prior work has been done to compare KX2-391 in relevant preclinical mouse models. As we have extensively examined dasatinib in orthotopic models for prostate cancer (11), and Phase I/II trials with dasatinib have shown great promise (Yu et al.), the goal of this work was to perform a head-on-head comparison between dasatinib and KX2-391 in this very relevant system.

In this study, we determined the efficacy of KX2-391 in inhibiting the primary prostate tumor growth and development of lymph node metastasis *in vivo*, compared to dasatinib. We demonstrated that 10mg/kg/day KX2-391 (p.o., b.i.d.) treatment significantly reduced the primary tumor weight (compared to the control group, $P=0.016$, Mann-Whitney U test) as well as incidence of lymph node metastasis (compared to the control group, $P=0.015$, Fisher's exact test). Compared to the dasatinib group, KX2-391 treatment (high dose) did not show significant differences in both tumor weight and in

incidence of lymph node metastasis. However, the median tumor weight and inter-quartile range of tumor weight in the high dose KX2-391 group was slightly less than dasatinib group, suggesting that 10mg/kg/day KX2-391 treatment may show higher potency in further animal experiments with larger sample size. Low dose KX2-391 (5mg/kg/day, p.o., b.i.d.) treatment had some effects in reducing both tumor weight and incidence of lymph node metastasis, but the effects were not statistically significant, compared to the control group or high dose KX2-391 groups. In both high and low concentrations, animals did not show particular adverse reactions to KX2-391. Therefore, we conclude that KX2-391 (10mg/kg/day) is equally potent in this preclinical prostate cancer murine model, suggesting that KX2-391 may be an alternative approach to those patients who show adverse reaction to dasatinib (e.g. allergy, pleural effusion), or those who do not respond to dasatinib while expressing activated Src in the biopsy tissues. Future analyses, such as cDNA array studies, would be important to determine the spectrum of inhibition between dasatinib and KX2-391 (i.e. if there is a different profile of targets affected directly and indirectly).

Interestingly, immunohistochemical analysis of tumor tissues demonstrated that KX2-391, targeting a peptide binding domain of SFKs, effectively suppressed activating phosphorylation of SFKs *in vivo* (**Figures 2 and 3**). The peptide substrate binding domain is presumed to be formed only when SFKs complex with binding partners in cells, i.e. when Src is activated. Accordingly, KX2-391 showed no ability to inhibit SFK or other purified kinases in *in vitro* kinase assays (Gao *et al.*, Kinex Pharmaceuticals, personal communications; manuscript submitted). This observation fits the concept that peptide binding domain inhibitors will block newly activated SFKs whereas ATP-competitive inhibitors inhibit both previously and newly activated SFKs. Indeed, a higher concentration of KX2-391 (23nM) than dasatinib (4nM) is required to achieve similar growth inhibitory effects on NIH3T3 cells transfected with constitutively-activated Src (Gao *et al.*, Kinex Pharmaceuticals, personal communications; manuscript submitted). However, interestingly, in a low serum condition (0.5% serum), lower concentration of KX2-391 (6.5nM) showed the similar growth inhibitory effects (50% growth inhibition) as dasatinib (6nM), supporting that a peptide-binding domain inhibitor is more effective against activated SFKs than inactive status (Gao *et al.*, Kinex

Pharmaceuticals, personal communications; manuscript submitted). In this study, IHC data of our work shows that KX2-391 inhibits autophosphorylation of SFK in highly metastatic tumor tissues, indicating that KX2-391 somehow affects autophosphorylation of SFKs in *in vivo* tumor tissue with presumably high basal Src activity level. This result may be explained, in part, because autophosphorylation of Tyr-419 residue is, in fact, achieved by trans-phosphorylation of Src molecules (23). Therefore, the peptide binding domain blockade may interrupt Src trans-phosphorylation, resulting in decreased phosphorylation at Tyr-419.

Furthermore, analysis of IHC staining of tumor tissues demonstrated that low dose KX2-391 (5mg/kg/day) more profoundly inhibited autophosphorylation of SFKs *in vivo* than did high dose treatment ($P < 0.01$, Student's t-test). One possible explanation for this observation is that while the majority of responding tumor cells were killed by the drug treatment, the remaining non-responder cells (i.e. cells potentially resistant to the inhibitor in the tissue actively grow). This possibility could be tested by re-culturing the tumor cells remaining in mice treated with higher dose KX2-391. Nonetheless, both the primary tumor size as well as development of lymph node metastasis were significantly suppressed in high dose-treated group, suggesting that the higher expression level of phospho-Src in the high dose group may reflect the consequence of the treatment. Furthermore, inhibition of PCNA-positive tumor cell percentage was dose-dependent (**Figure 4**), suggesting that, together with the *in vivo* tumor data in **Table 1**, biological effects of SFK inhibition in this preclinical model correlate with dose.

In conclusion, we demonstrated that KX2-391, a novel SFK inhibitor targeting peptide substrate binding domain, showed similar efficacy in suppressing the primary tumor growth as well as preventing development of the lymph node metastasis as dasatinib in an *in vivo* prostate cancer mouse model. Our data suggest that a novel SFK inhibitor can be an alternative approach for the patients who show adverse reactions to the pre-existing SFK inhibitors.

Table 1. Effects of KX2-391 and dasatinib on Growth and Lymph Node Metastasis of PC-3MM2GL Cells in an Orthotopic Nude Mouse Model

Treatment Group	Tumor Incidence	Tumor weight (g)		LN metastases Incidence ⁽⁶⁾
		Median ⁽¹⁾	(IQR) ⁽⁵⁾	
Control	5/6	2.27	(2.61~1.98)	5/5
KX2-391 5.0mg/kg/day	5/6	1.16 ⁽²⁾	(1.28~0.94)	2/5
KX2-391 10.0mg/kg/day	5/6	0.35 ⁽³⁾	(0.56~0.24)	1/5 ⁽⁷⁾
Dasatinib 15mg/kg/day	5/6	0.43 ⁽⁴⁾	(1.34~0.30)	1/5 ⁽⁸⁾

- (1) Median tumor weights among four groups were significantly different by multiple group comparison ($P=0.032$, by Kruskal-Wallis test).
- (2) Median tumor weight in the 5.0mg/kg/day KX2-391 group was not significantly different from the control group ($P=0.076$, by Mann-Whitney U test).
- (3) Median tumor weight in the 10.0mg/kg/day KX2-391 group was significantly decreased, compared to the control group ($P=0.016$, by Mann-Whitney U test).
- (4) Median tumor weight in the dasatinib group was significantly decreased, compared to the control group ($P=0.028$, by Mann-Whitney U test). However, there was no statistical significance between 10mg/kg/day KX2-391 and dasatinib groups.
- (5) IQR - inter-quartile range, which represents a range of 75th percentile and 25th percentile of the tumor weight in the group.
- (6) Incidence of lymph node metastases was determined by identifying solid, opaque and enlarged iliac lymph node(s).
- (7) and (8) Incidences of lymph node metastasis were significantly decreased ($P=0.015$, by Fisher's exact test) both in KX2-391 10mg/kg/day and dasatinib groups, compared to the control group.

FIGURE LEGENDS

Figure 1 Effects of KX2-391 and Dasatinib on the Primary Tumor Weight in PC-3MM2GL Orthotopic Nude Mouse Model

PC-3MM2GL cells were injected into six animals per group, and treated with control placebo, low (5mg/kg/day) or high (10mg/kg/day) dose KX2-391, and dasatinib (15mg/kg/day). After four weeks' treatment, mice were euthanized and tumors were surgically excised and weighed. Each dot represents individual tumor weight and boxes show inter-quartile range. Highest, lowest and median weights in each group were indicated by horizontal lines.

Figure 2 Immunohistochemistry of Tumor Tissues

Three tumors that weighed close to the median tumor weight in each group were selected for immunohistochemical staining. Serially sectioned slides were stained for hematoxylin and eosin, total Src, total Lyn and autophosphorylated SFKs. Staining was performed as described in the Materials and Methods. Representative area-matched images are represented in the panel.

Figure 3 Quantification of Immunohistochemical Staining Intensities of autophosphorylated SFKs

Staining intensities of IHC for autophosphorylated SFKs in three medium-sized tumors from each group were quantified as described in the Materials and Methods. Five randomly selected areas per sample were photographed. DAB-color extraction and quantification were performed, and staining intensities were divided by area resulting in staining intensity in arbitrary unit. *Bars* represent average of staining intensities in each group, and *error bars* represent standard deviation. Statistical difference of averages was calculated by One-way ANOVA ($P < 0.01$). Statistical significances between selected groups were described in the text.

Figure 4 Proliferating Cell Nuclear Antigen Staining

Frozen tumor sections were stained for proliferating cell nuclear antigen (PCNA). **A.** Bright field microscope images (100× magnification) were photographed and analyzed to quantify PCNA-positive cell percentage. *Bars* represents average percentage of PCNA-positive cells counted in three randomly selected fields in three tumor samples from each group. *Error bars* show the standard deviation. ($P < 0.01$ by One-way ANOVA) **B.** Representative images in each group are shown.

Figure 5 CD31 Microvessel Staining

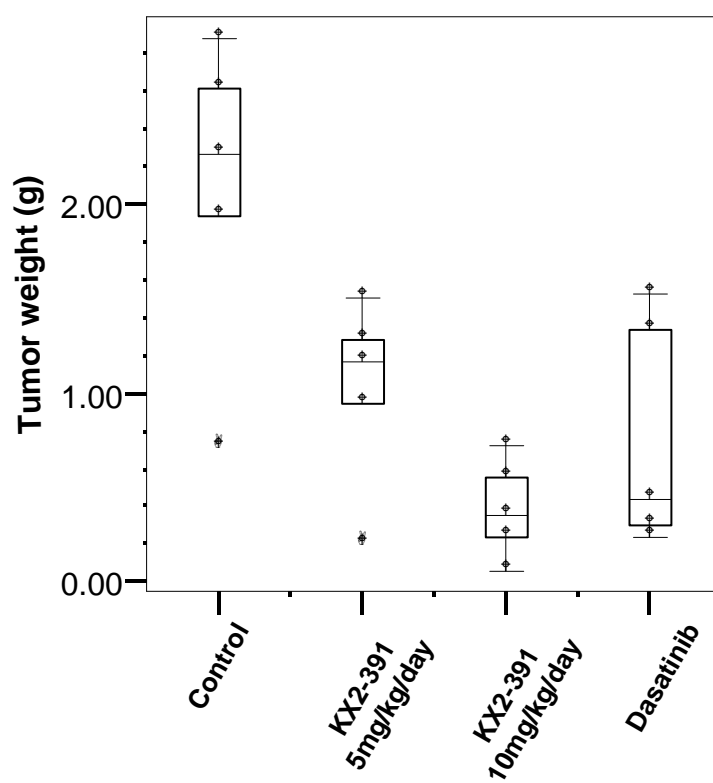
Frozen tumor sections were stained for mouse CD31 to quantify microvessel density of tumor tissues. Bright field microscope images (100× magnification) were taken and positively stained microvessels were counted. Representative images are shown.

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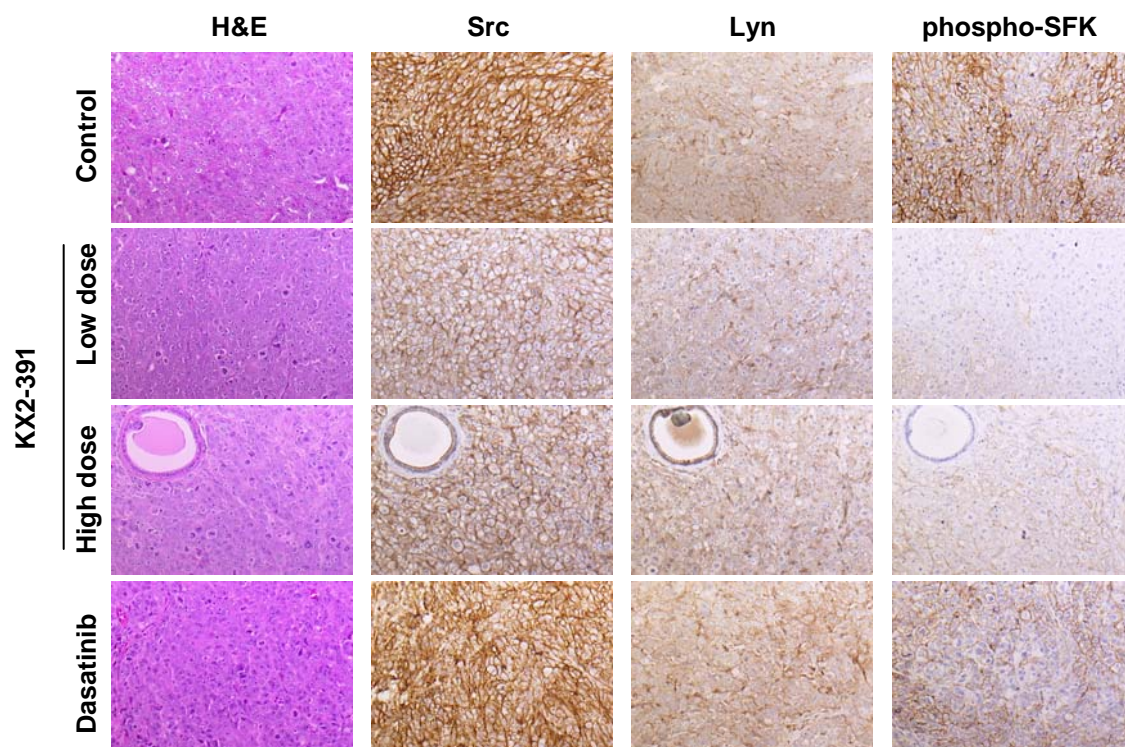
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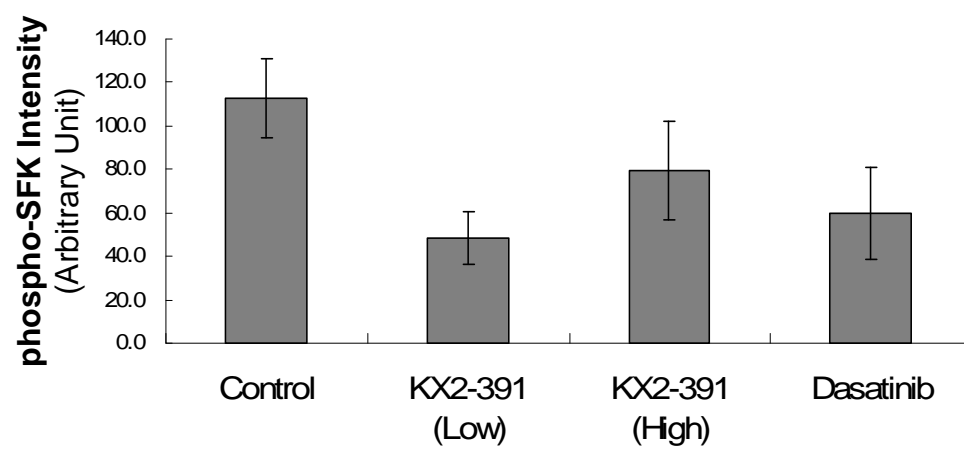
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Figure 1



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Figure 2

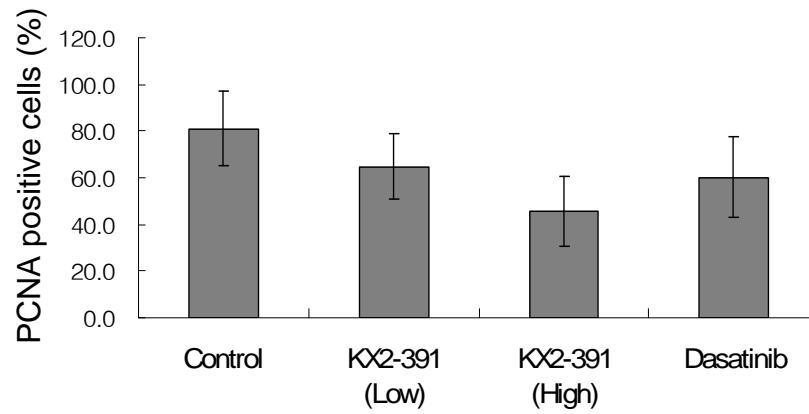


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Figure 3

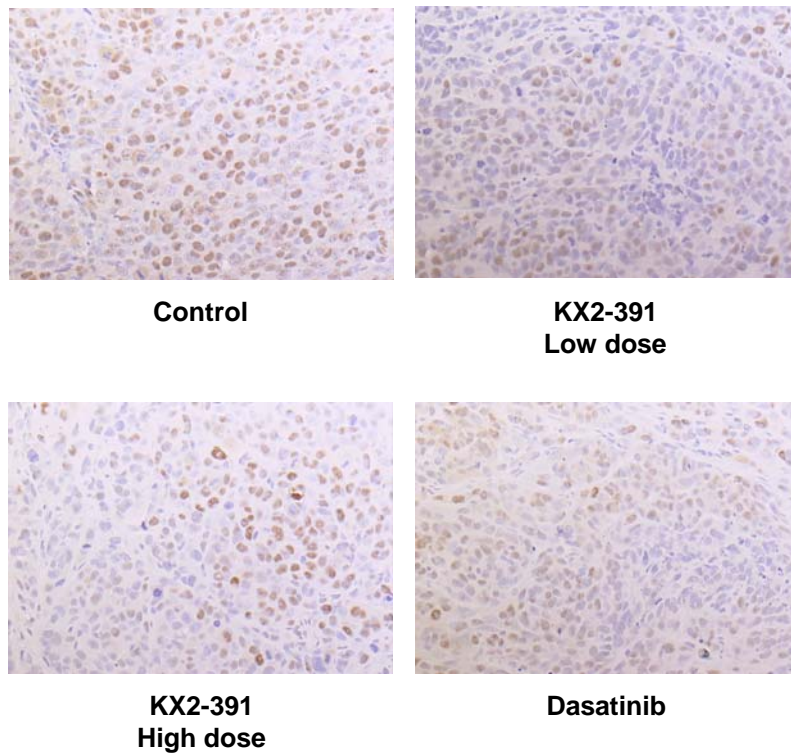


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Figure 4

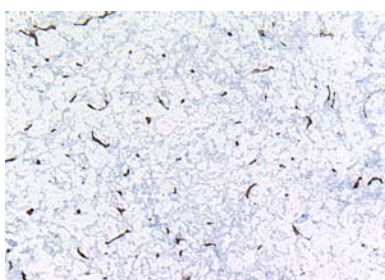
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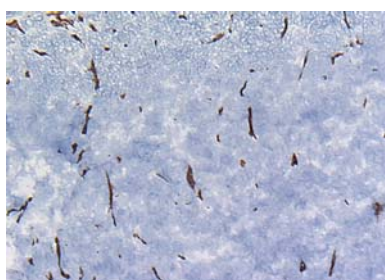
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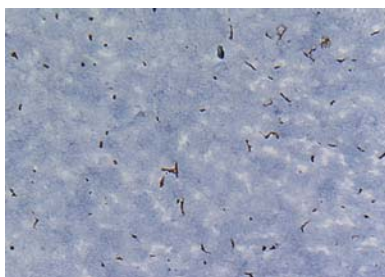
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Figure 5



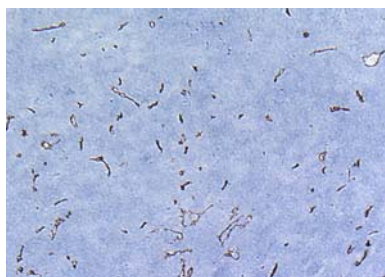
Control



**KX2-391
Low dose**



**KX2-391
High dose**



Dasatinib